MOLECULAR AND CELLULAR BIOLOGY, Oct. 1995, p. 5461–5469 Vol. 15, No. 10
0270-7306/95/$04.00+0

Mutations on the DNA-Binding Surface of TATA-Binding Protein Can Specifically Impair the Response to Acidic Activators In Vivo

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Received 18 May 1995/Returned for modification 22 June 1995/Accepted 11 July 1995

The TATA-binding protein (TBP) contains a concave surface that interacts specifically with TATA promoter elements and a convex surface that mediates protein-protein interactions with general and gene-specific transcription factors. Biochemical experiments suggest that interactions between activator proteins and TBP are important in stimulating transcription by the RNA polymerase II machinery. To gain insight into the role of TBP in mediating transcriptional activation in vivo, we implemented a genetic strategy in *Saccharomyces cerevisiae* that involved the use of a TBP derivative with altered specificity for TATA elements. By genetically screening a set of TBP mutant libraries that were biased to the convex surface that mediates protein-protein interactions, we identified TBP derivatives that are impaired in the response to three acidic activators (Gcn4, Gal4, and Ace1) but appear normal for constitutive polymerase II transcription. A genetic complementation assay indicates that the activation-defective phenotypes reflect specific functional properties of the TBP derivatives rather than an indirect effect on transcription. Surprisingly, three of the four activation-defective mutants affect residues that directly contact DNA. Moreover, all four mutants are defective for TATA element binding, but they interact normally with an acidic activation domain and TFIIB. In addition, we show that a subset of TBP derivatives with mutations on the DNA-binding surface of TBP are also compromised in their responses to acidic activators in vivo. These observations suggest that interactions at the TBP-TATA element interface can specifically affect the response to acidic activator proteins in vivo.

The mechanisms by which transcriptional activator proteins stimulate the RNA polymerase II (Pol II) machinery have been intensively investigated in vitro. Activators appear to function by affecting one or more steps in the assembly of general transcription factors into a preinitiation complex (5, 11, 14, 21, 32, 33, 47, 50). In principle, activators might recruit general factors to the promoter, stabilize protein-protein interactions within the preinitiation complex, and/or facilitate conformational changes in initiation factors. In vitro, activators can interact directly with the TATA-binding protein (TBP) (19, 42), TBP-associated factors (TAFs) that are components of the TFIID complex (11, 14), TFIIA (36), TFIIB (34), TFIIF (21), TFIIH (54), and Pol II holoenzyme (13). In particular, many regulatory proteins such as VP16 (19, 42), p53 (39), and E1A (30) can interact with TBP. In addition, TBP mutants that are defective for activated transcription in vitro have impaired interactions with activation domains, TFIIA, TFIIB, and DNA (24). The physiological significance of these results remains to be clarified.

The molecular mechanisms of transcriptional activation in vivo are poorly understood. In yeast cells, activators can stimulate transcription by increasing recruitment of TBP to the promoter. First, accessibility of TBP to the *his3* TATA element was shown to be a rate-limiting step that could be accelerated by the Gcn4 acidic activation domain (27). Second, direct recruitment of TBP to the promoter by physically connecting TBP to heterologous DNA-binding domains activates transcription (3, 26). Transient-transfection experiments in mammalian cells also indicate that TBP plays a role in transcriptional activation (6, 46). The hypothesis that activators stimulate TBP recruitment in vivo is attractive in light of observations in vitro that TBP binds very poorly to TATA elements in the context of chromatin (18, 53). What remains unclear, however, is the mechanism by which activators recruit TBP or other basal factors to the promoter.

To address this question, we have undertaken a genetic strategy to isolate yeast TBP mutants that are defective in the response to acidic activators but are otherwise normal for Pol II transcription in vivo. It is important to note that the above definition of activation-defective mutants does not involve the concept of basal transcription as defined in vitro. In principle, the basal transcription reaction involves the minimal set of purified general factors that is sufficient to direct accurate initiation from a promoter containing only a TATA and initiator element. In vivo, it is obviously impossible to generate such a situation because the cell contains all proteins involved in transcription (including activators) at physiological levels, and because chromosomally located promoters will always have sequences upstream of the TATA element that are potential weak binding sites for activator proteins. Thus, it cannot be determined whether the low level of transcription that is typically observed in vivo from promoters lacking upstream elements (45) reflects basal transcription as defined in vitro or is due to unknown activators binding to cryptic sequences upstream of the TATA element. Similarly, it cannot be determined whether transcription in the absence of a known activator reflects basal transcription or involves an unknown activator. For these reasons, we define an activation-defective TBP derivative as having a defect in the response to multiple acidic activators but conferring normal levels of transcription from other Pol II promoters in vivo.

Our strategy exploits the unique property of an altered-specificity TBP derivative (TBP^{m3}) to bind and function at

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promoters bearing the mutant TATA element, TGTAAA (43). TBP contains a concave surface that interacts with TATA promoter elements and a convex surface that mediates interactions with general and gene-specific transcription factors (23, 25). Because most models for activation invoke protein-protein interactions between activators and general factors, we screened TBP^{m3} mutant libraries that were biased to the convex surface. In this report, we characterize TBP derivatives that are specifically defective in the response to acidic activators. Surprisingly, most of the activation-defective TBP mutants affect residues that directly contact DNA, and all of them are unable to bind TATA elements in vitro. TBP derivatives, previously characterized as having DNA-binding and transcriptional activation defects in vitro (24), are also compromised in their responses to acidic activators in vivo. These results suggest that interactions at the TBP-TATA element interface can specifically affect transcriptional activation.

MATERIALS AND METHODS

Strains and DNAs. *Saccharomyces cerevisiae* yML1 was derived from Δ*CUP1*-51 (31) by gene replacement of the *HIS3* locus with Sc3765 (12), which contains a TGTAAA TATA element and no activator-binding site, and the introduction of Ycp88-GCN4 (16), a plasmid constitutively expressing Gcn4, and a second plasmid containing a fusion of the *HIS3* promoter and N-terminal 103 amino acids from Sc3745 (12) to the *CUP1* coding sequence. The *CUP1* structural gene was isolated as a *Sma*I-*Kpn*I fragment derived from plasmid pBS-*CUP1-Sma* (31). Analysis of mutants obtained in the TBP^{m3} context was performed in strains derived from KY320 (4) in which the *HIS3* locus was replaced with TGTAAA-containing alleles Sc3765 (no Gcn4-binding site; strain yML2), Sc3745 (with a Gcn4-binding site; strain yML3), or Sc3660 (four Gal4-binding sites; strain yML4). Analysis of mutants in the wild-type TBP context was performed by plasmid shuffling into strain BYΔ2 (7). To assess Ace1 activation, the *HIS3* locus of BYΔ2 was replaced with a derivative that contains a single Ace1-binding site upstream of the TATAAA TATA element (20) to give strain yML6. For the genetic complementation experiment detailed in Fig. 5, the *HIS3* locus of BYΔ2 was replaced with allele Sc3660 (4) to generate strain yML7.

TBP libraries generated by regional codon randomization (9) were reconstructed in the TBP^{m3} context as follows. Libraries N1, N2, and N3 were converted by subcloning an *Xba*I-*Bam*HI fragment containing the three mutations conferring altered specificity (43). Libraries N4, N5, and N6 were constructed by PCR (9) using TBP^{m3} as a template. As before, TBP libraries were carried on *TRP1*-marked centromeric plasmids. Separation of the double mutant (S118L, S128T) was achieved by subcloning the *Age*I-*Bam*HI fragment of the double mutant into the parental TBP^{m3} context (to give S128T) and subcloning the same fragment from parental TBP^{m3} into the double mutant (to give S118L). Generation of the TBP mutants L114K, L189K, and K211L was accomplished by PCR mutagenesis using the megaprimer method (38) on a TBP or TBP^{m3} template. TBP mutants K110L and K120L were generated by subcloning of the *Xba*I-*Bam*HI fragment of TBP^{m3} as described above.

Screen for activation-deficient TBPs. Strain yML1 was transformed with TBP^{m3} libraries and plated on media selecting for the library plasmid. Transformants (approximately 5,000 from each library) were replica plated onto synthetic minimal medium lacking histidine and containing 0.5 mM aminotriazole (AT) to assay expression from the nonactivated promoter and onto synthetic minimal medium containing histidine and 160 μM $CuSO_4$ to monitor transcription from the Gcn4-activated reporter. Candidates from this screen demonstrating reasonable growth on the AT plate and poor growth on the $CuSO_4$ plate were selected for further analysis. Plasmids encoding TBP^{m3} derivatives were rescued from these candidates and passaged through *Escherichia coli*, and the resulting DNA was retransformed into strains yML2 and yML3 to confirm the phenotype.

Phenotypic analysis. Plate phenotypes for strains containing TGTAAA reporters and altered-specificity TBP derivatives were determined by a spotting assay. Yeast strains grown to late log phase in appropriate media (as specified) were harvested by centrifugation and resuspended in a buffer containing 10 mM Tris (pH 8.0) and 5 mM EDTA, a buffer that minimizes aggregation (48); 10^5 cells for each strain were spotted onto synthetic minimal media with compositions as described in the figure legends. To assay Gal4 responses for TBP^{m3} derivatives, strains were grown in Casamino Acids medium containing raffinose (nonrepressing) as the sole carbon source. Viability of yeast TBP derivatives in the wild-type DNA-binding specificity context was assayed by plasmid shuffling (7). Gal4-dependent activation was determined by transforming strains with YCp86-Sc3801 (41), which contains four Gal4-binding sites upstream of the *his3* TATA region and *his3-lacZ* structural gene. Cells were grown to mid-log phase in synthetic minimal medium containing raffinose (nonrepressing) as the sole carbon source and subsequently split into medium containing either glucose (repressing) or galactose (inducing) as the sole carbon source. Cultures were then harvested at mid-log phase and assayed for β-galactosidase activity.

RNA analysis. For analysis of Ace1 activation, yML6 derivatives supported by wild-type or mutant TBPs were grown in synthetic minimal glucose medium to mid-log phase and split into two series of parallel cultures in fresh medium. When these cultures reached mid-log phase, one series of cultures was induced with 200 μM $CuSO_4$ for 1 h, at which point both series of cultures were harvested for RNA analysis. For the genetic complementation assay, strains supported by the wild-type and mutant TBP derivatives were transformed with TBP^{m3} and grown in a Casamino Acids medium containing raffinose (nonrepressing) as the sole carbon source. Cultures were grown to mid-log phase and split into Casamino Acids medium containing either glucose (repressing) or galactose (inducing) as the sole carbon source and harvested at mid-log phase. In all cases, RNA levels were measured quantitatively by S1 nuclease analysis using oligonucleotides probes (8, 28).

In vitro analysis of TBPs. TBP derivatives were cloned into the *Nde*I and *Bam*HI sites of vector PET-15b (Novagen) as follows. An *Nde*I-*Bgl*II fragment containing the N terminus of yeast TBP (7) was ligated to the *Bgl*II-*Bam*HI fragment of the TBP derivatives containing the rest of the TBP coding sequence, with destruction of the *Bam*HI site. All mutations analyzed were present between the *Bgl*II (within the TBP coding sequence) and *Bam*HI sites of TBP. TBPs were purified as instructed by the manufacturer (Novagen). Proteins thus purified were estimated to be greater than 50% pure by Coomassie blue staining. Concentrations were determined by the Bradford assay and calculated for wild-type TBP. Protein concentrations for the TBP mutants were estimated by Coomassie blue staining, using serial dilutions of wild-type TBP as a standard. Gel mobility shift experiments were performed by incubating a 45-bp fragment (0.5 ng) containing the adenovirus E1B TATA box with the indicated amounts of the TBP derivative in the presence of 200 ng of poly(dG-dC), 100 mM KCl, 40 mM *N*-2-hydroxyethylpiperazine-*N*′2-ethanesulfonic acid (HEPES; pH 7.9), 20 mM Tris (pH 7.5), 5 mM $MgCl_2$, 10% glycerol, 1 mM dithiothreitol, and 0.5 mM phenylmethylsulfonyl sulfate. TBP complexes were electrophoretically separated on 4% native polyacrylamide gels run in a Tris-glycine buffer system supplemented with 4 mM $MgCl_2$. Column association experiments involved incubating 20 μl of glutathione-agarose beads containing glutathione *S*-transferase (GST), GST-VP16, and GST-TFIIB with TBP derivatives for 1 h in 100 μl of buffer containing 100 mM KCl, 20 mM HEPES (pH 7.9), 5 mM $MgCl_2$, 20 μg bovine serum albumin, 0.2 mM EDTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 10% glycerol, and 0.03% Nonidet P-40. The beads were washed five times with 200 μl of buffer, and bound proteins were eluted in 40 μl of sodium dodecyl sulfate-polyacrylamide gel electrophoresis loading buffer and separated by electrophoresis. TBP bound to the various columns tested was visualized by Western blotting (immunoblotting) with an anti-TBP antibody.

RESULTS

A genetic screen for isolating activation-deficient derivatives of TBP. To obtain TBP mutants with defects in activated transcription, we exploited the unique property of an altered-specificity TBP derivative (TBP^{m3}) to bind and function at promoters bearing the mutant TATA element, TGTAAA (43). TBP^{m3} versions of yeast and human derivatives have been examined for activation in yeast and human cells (22, 43, 46). A yeast genetic screen performed in the context of TBP^{m3} has several advantages. First, because the screen is carried out in the presence of wild-type TBP, mutations that adversely affect both activation and viability are not lost. Second, TBP^{m3} is required for transcription from TGTAAA-containing promoters but not from native yeast promoters; this reduces the likelihood of isolating activation-deficient TBP mutants that indirectly generate their phenotypes by altering expression of genes other than the reporter. Third, the availability of genetic screens makes it possible to examine many TBP derivatives for the desired properties.

The directed genetic screen for activation-defective TBP derivatives is diagrammed in Fig. 1. Six highly compact and complex libraries generated by regional codon randomization (9) were reconstructed in the TBP^{m3} background. These libraries are strongly biased to the convex surface of TBP that mediates protein-protein interactions; of the 95 residues mutated, only 14 are located on the concave, DNA-binding surface. The libraries were introduced into strain yML1, which contains *his3* promoter derivatives containing the TGTAAA TATA element fused to selectable structural genes. The non-

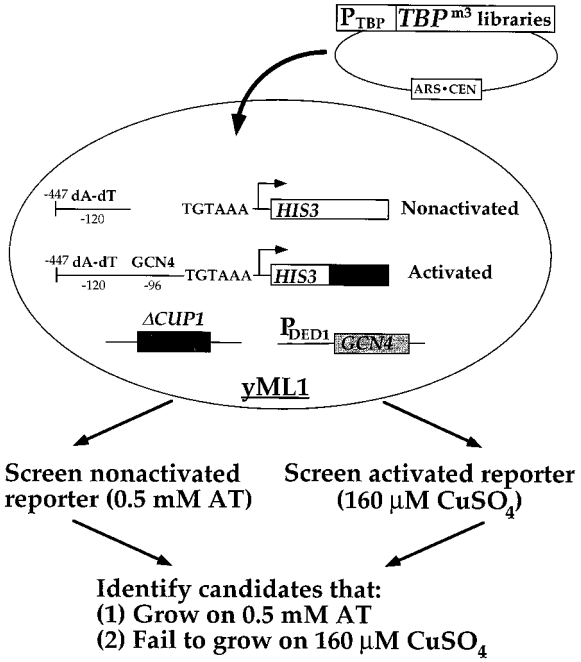


FIG. 1. Genetic screen for activation-deficient TBP mutants. Libraries generated by regional codon randomization (9) were reconstructed in the altered-specificity context (TBP^{m3}) and introduced into strain yML1, which contains the following elements: a nonactivated, TGTAAA-driven reporter (*HIS3*); a GCN4-activated, TGTAAA-driven reporter (*CUP1*); and a constitutive *GCN4* expression plasmid (P_{DED1}-GCN4). TGTAAA promoters for both reporters are *HIS3* promoter derivatives containing a deletion of T_C (12). Individual transformants were screened as described.

activated promoter drives expression of *HIS3*, which can be monitored by resistance to AT, a competitive inhibitor of the *HIS3* gene product. The Gcn4-activated promoter directs expression of *CUP1*, which can be monitored by resistance to exogenous copper sulfate in the growth medium (31). The promoters for the two reporters are identical in all respects except that one contains a Gcn4-binding site while the other does not. In comparison with the parental TBP^{m3}, an activation-deficient mutant should show high activity from the nonactivated reporter but low activity from the activated reporter.

Isolation of a rare class of TBP mutants that is impaired in transcriptional activation. Approximately 5,000 transformants from each TBP^{m3} library were screened on medium containing either 0.5 mM AT or 160 μM $CuSO_4$. Colonies that grew fairly well on AT but poorly on $CuSO_4$ were selected, and the plasmids encoding the TBP^{m3} derivatives were retransformed into a isogenic yeast strains carrying the nonactivated and Gcn4-activated promoters fused to the *HIS3* structural gene. Figure 2A shows a graphical representation of the range of mutant phenotypes in which growth on AT for a given TBP^{m3} derivative in either the nonactivated or Gcn4-activated reporter strain was scored on an arbitrary scale with wild-type TBP at the bottom and TBP^{m3} at the top. As expected, defects in activated transcription are strongly correlated with defects in transcription from the nonactivated promoter. However, within one class of mutants having a modestly decreased level of nonactivated transcription (Fig. 2A, boxed), we observed a wide distribution of phenotypes on the activated promoter.

From this class of TBP^{m3} mutants having equivalent phenotypes on the nonactivated promoter, four TBP^{m3} derivatives exhibiting the lowest level of Gcn4-activated transcription were selected for further analysis (Fig. 2A, circled). Three of these mutants contain single amino acid substitutions (N159L, V161A, and F148L); the remaining mutant contained two changes (S118L and S128T). Compared with the parental TBP^{m3}, all four mutants exhibit only slightly reduced levels of activity on the nonactivated promoter, whereas they show dramatic effects on the Gcn4-activated promoter (Fig. 2B). In contrast, TBP^{m3} derivatives containing K145G, R137S, A140C plus Q144L, and S136M, which display similar functions on the nonactivated promoter, give levels of activation more characteristic of the general distribution of mutants. Separation of the S118L,S128T double mutant indicates that S118L is responsible for the activation-defective phenotype; S128T behaves indistinguishably from the parental TBP^{m3}. Double mutants representing the six pairwise combinations of the activation mutations were equally or more impaired in the ability to support Gcn4-dependent activation compared with the corresponding single mutants (Fig. 2B).

We tested the four TBP^{m3} derivatives for Gal4-dependent activation by introducing them into a strain carrying a TGT AAA-containing promoter with four binding sites for the Gal4 acidic activator (Fig. 2C). In comparison with parental TBP^{m3}, the four TBP^{m3} derivatives are impaired in the ability to support Gal4-dependent activation, although to different extents. The S118L, F148L, and V161A derivatives show very little activity on this Gal4-dependent promoter, whereas N159L is only partially defective.

Activation defects are retained in the context of wild-type TBP. To verify that the activation-deficient phenotypes were not an artifact of the TBP^{m3} context, we introduced the mutations into the context of wild-type TBP. Strains containing the four derivatives as the sole source of TBP grow well at 30°C, with little or no effect on doubling time in YPD medium. At 37°C, strains containing S118L, N159L, and V161A exhibit slightly decreased growth compared with strains containing F148L or wild-type TBP. Growth rates of double mutant strains were equal to or slightly slower than those of the corresponding single-mutant strains. As determined by Western blotting, TBP levels in the mutant strains were indistinguishable from that of the wild-type strain (see Fig. 4B).

To test whether these mutant strains were deficient for activation by Gal4 and Ace1, we generated strains containing appropriate reporter constructs with a wild-type (TATAAA) element. In all four mutant strains, β-galactosidase activity from the Gal4-dependent reporter under activating conditions (in galactose) varied between 6 and 24% of the activity of the wild-type strain (Fig. 3A); in the double-mutant strains, activities ranged from 6 to 12% (Fig. 3B). As expected, the behaviors of the activation-deficient TBPs in this assay paralleled their activities in the TBP^{m3} context (compare Fig. 2C with Fig. 3A). With the exception of N159L, which was least affected for Gal4 activation, the mutant TBP derivatives were also impaired in transcriptional activation by Ace1 (Fig. 3C). V161A was very deficient (about 5% of wild-type activity), whereas S118L and F148L showed about 25% of wild-type activity. The apparent defects observed in the absence of copper are Ace1 dependent (10) and do not represent defects in nonactivated transcription.

TBP derivatives do not affect Pol I, Pol III, and constitutive Pol II transcription. In the TBP^{m3} context used in their isolation, the activation-deficient derivatives appear to be slightly defective for nonactivated transcription (Fig. 2A and B). However, this apparent defect may be due not to the S118L, F148L, N159L, and V161A mutations per se but rather to the fact that they are analyzed in the context of TBP^{m3}, which contains three mutations on the DNA-binding surface that mediate the altered TATA element specificity. We therefore analyzed the

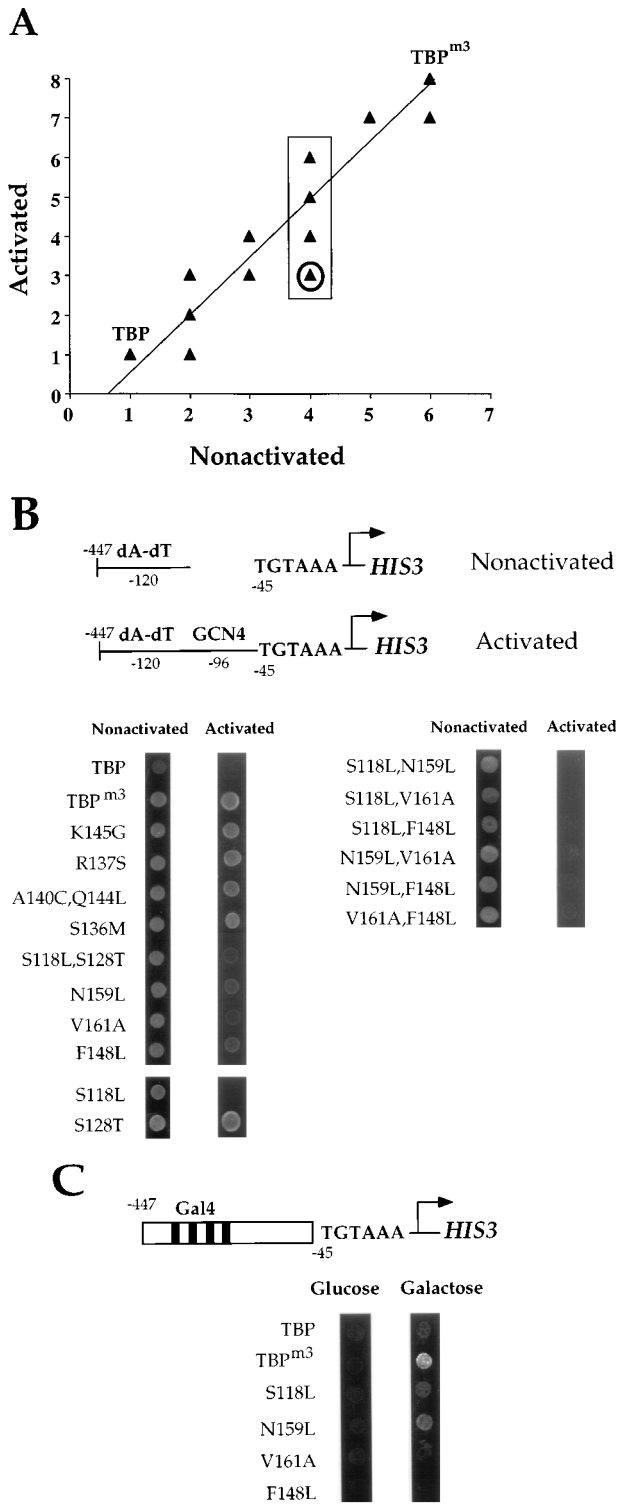


FIG. 2. Isolation of activation-deficient TBP mutants. (A) Phenotypes obtained from the screen. Mutants were scored for activity from each reporter, using an arbitrary scale (phenotypes conferred by wild-type TBP and the parental TBP^{m3} were set at the bottom and top of the scale, respectively). Each point on the graph denotes a phenotypic class and represents one or more mutant TBP derivatives. Boxed, series of phenotypic classes with equivalent function on the nonactivated promoter; circled, class of mutants defined to be activation deficient. (B) Phenotypic assays for nonactivated and Gcn4-activated *HIS3* expression conferred by mutant TBP^{m3} derivatives. The *HIS3* promoter derivatives are identical to those depicted in Fig. 1 except that the activated reporter directs expression of *HIS3*. Mutants were introduced into strain yML2 (nonactivated reporter) or yML3 (activated reporter), and the resulting cells were spotted on plates containing either 0.5 mM AT (nonactivated) or 5.0 mM AT (activated). Mutants K145G, R137S, A140C,Q144L, and S136M are activation-competent mutants whose activities on the nonactivated promoter are similar to those of the activation-deficient mutants S118L,S128T, N159L, V161A, and F148L. Individual amino acid changes in S118L,S128T were tested separately to determine the basis for the activation-deficient phenotype. Analysis of the six possible double mutants between S118L, N159L, V161A, and F148L constructed in TBP^{m3} is shown at the right. (C) Responses of TBP mutants to the Gal4 activator. TBP^{m3} derivatives were introduced into strain yML4 (Gal4-dependent TGTAAA-containing *HIS3*, with four Gal4-binding sites denoted by black boxes), and the resulting strains were tested for growth in medium containing either glucose or galactose as the carbon source and 5 mM AT.

mutations in the context of wild-type TBP (same strains as used for Fig. 3) for transcription from representative Pol I (rRNA), Pol III (tRNA-I), TATA-less Pol II (*TRP3*), and constitutive Pol II (*DED1* and *RPS4*) promoters. For all five genes tested, transcription in the mutant strains was similar to that observed in the wild-type strain (Fig. 4). Although basal transcription as defined in vitro cannot be measured in vivo (see the introduction), these results indicate that the TBP mutations do not generally impair Pol II transcription. Thus, in the context of wild-type TBP, the mutant proteins are specifically defective in the response to acidic activators in vivo.

A genetic complementation experiment verifies the primary nature of activation deficiency. There are two mechanisms by which a TBP mutant might generate an activation-deficient phenotype. In the desired case, TBP mutants specifically and directly affect the process of activation. Alternatively, as TBP plays an essential role in all transcriptional events in vivo, an activation-deficient phenotype on a particular reporter might represent an indirect effect due to the altered expression of a gene(s) important for activation. For example, TBP derivatives defective in the synthesis of specific activator proteins, TAFs, or other coactivators might lead to the appearance of an activation-defective phenotype.

We have designed a genetic complementation experiment to distinguish between these possibilities (Fig. 5A). Strain yML7 contains a Gal4-dependent, TGTAAA-containing promoter that drives *HIS3* expression and is supported by wild-type TBP on a *URA3*-marked plasmid. The four TBP mutants (in the wild-type context) were introduced into strain yML7 by plasmid shuffling, and activation deficiency was confirmed by analysis with the Gal4-dependent *lacZ* reporter (data not shown). The resulting strains (and the wild-type TBP control) were transformed by a plasmid expressing the parental (i.e., otherwise wild-type) TBP^{m3}. If a TBP mutant indirectly generates an activation-deficient phenotype by affecting expression of gene(s) important for the activation process, then TBP^{m3} should also show an activation-defective phenotype on its cognate promoter. On the other hand, in a cell supported by a TBP mutant with a primary activation defect, TBP^{m3} should function normally in activated transcription from a TGTAAA promoter. Thus, in this genetic complementation experiment, TBP^{m3} serves as a probe for the activation competency of the cell.

In the control strain supported by wild-type TBP, TBP^{m3} drives *HIS3* transcription when the cells are grown in galactose (Fig. 5B). In strains supported by the S118L, N159L, V161A, and F148L derivatives, TBP^{m3} function varied between 80 and 100% of wild-type function. Thus, the activation-deficient phenotypes of the four TBP mutant strains are directly due to inherent functional defects in transcriptional activation.

Activation-deficient TBP alleles are defective for DNA binding. Mapping of the TBP mutations onto the X-ray structure of a TBP-TATA element complex (23, 25) reveals that three of

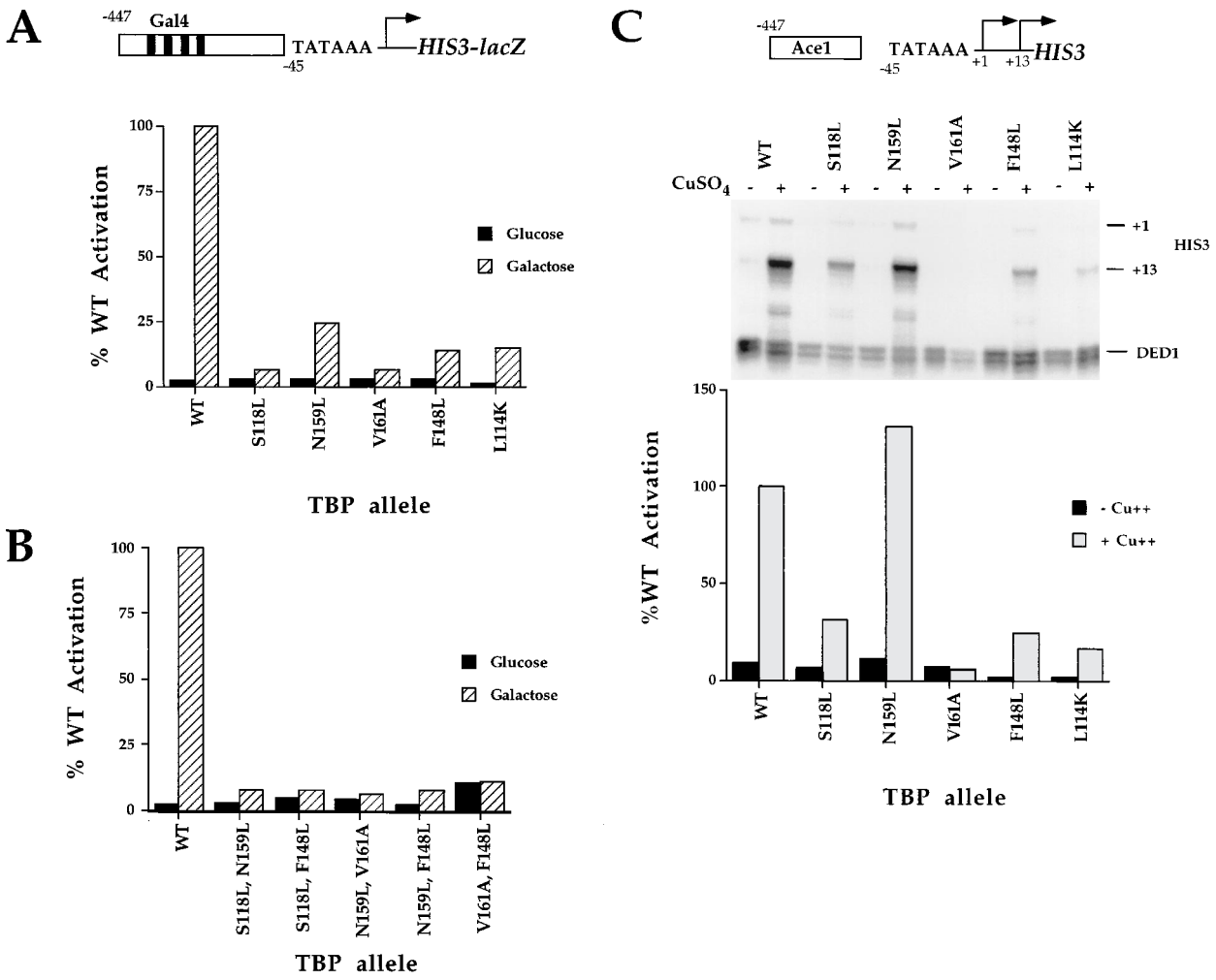


FIG. 3. Gal4 and Ace1 activation in strains supported by mutant TBPs. (A) Gal4 activation. Strains containing the indicated TBP derivatives (in an otherwise wild-type [WT] context) and a Gal4-dependent β-galactosidase reporter bearing a consensus TATAAA element (41) were grown in glucose or galactose medium. (B) Gal4 activation in strains supported by double mutants. The S118L,V161A double mutant did not support cell growth and hence was not tested. (C) Ace1 activation. Strains containing the indicated TBP derivatives (in an otherwise wild-type context) and an Ace1-dependent *HIS3* reporter bearing a consensus TATAAA element were grown in presence (+) or absence (−) of 200 μM $CuSO_4$ and assayed for *HIS3* and *DED1* RNA levels by S1 nuclease analysis. The level of the *HIS3* +13 transcript (normalized to the level of the *DED1* internal control) was quantitated by PhosphorImager analysis. Ace1-dependent transcription occurs in the absence of copper (10); hence, the apparent decrease in transcription in the mutant TBP strains in the absence of copper is Ace1 dependent and does not reflect a deficiency in nonactivated transcription.

the four TBP derivatives isolated here (S118L, N159L, and V161A) affect residues that directly contact DNA. Given that only 14 of the 95 residues represented in the screened libraries map to the DNA-binding surface, this result was very surprising ($P < 0.01$). This result was also surprising given that nearly all models of transcriptional activation involving TBP have emphasized protein-protein interactions mediated by the convex (i.e., non-DNA-binding) surface.

To test whether DNA binding was indeed affected, we expressed histidine-tagged TBP mutant proteins (in a wild-type context) in *E. coli* and purified them by nickel affinity column chromatography. As assayed by gel retardation, wild-type TBP bound specifically to a TATA-containing probe, whereas none of the mutants could bind detectably at similar TBP concentrations (Fig. 6A). Indeed, none of the mutants could bind detectably at 10-fold-higher protein concentrations, with the exception of F148L, which gave a very faint signal. Thus, the four activation-defective mutants possess DNA-binding capacities at least 100-fold below wild-type levels. Addition of TFIIA or TFIIB to the reaction does not rescue the DNA-binding defect (data not shown). The inability to bind TATA sequences is not due to a gross unfolding or inactivation of the *E. coli*-generated proteins. When tested for interactions with TFIIB or VP16, two factors with well-characterized associations with TBP, all four mutants behaved indistinguishably from wild-type TBP (Fig. 6B).

A subset of other mutations on the TBP DNA-binding surface confer activation defects in vivo. Three TBP mutants that support basal transcription but do not respond to the VP16 acidic activation domain have been previously identified by assays in vitro (24). While the mutations involved (L114K, L189K, and K211L) cause a variety of biochemical defects, they all affect residues that directly contact DNA. As most of the TBP derivatives described in the current study affect residues on the DNA-binding surface, we therefore examined whether these previously identified TBP mutants are defective for transcriptional activation in vivo.

We first tested whether the L114K, L189K, and K211L de-

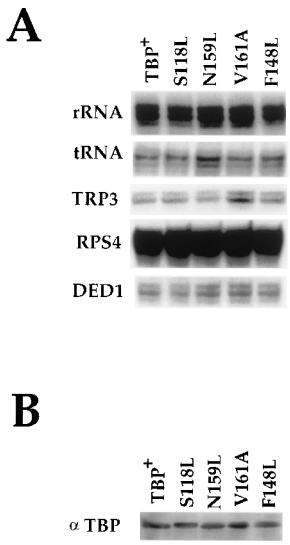
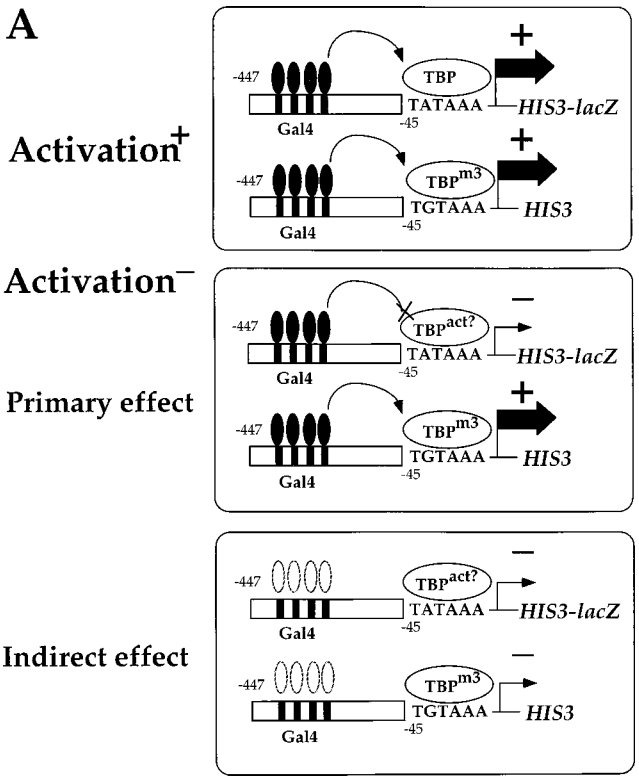


FIG. 4. Transcription from Pol I, Pol III, TATA-less Pol II, and constitutive TATA-containing Pol II genes. (A) Forty micrograms of total RNA harvested from strains supported by the indicated TBP derivatives was subjected to S1 nuclease analysis using probes directed against rRNA, tRNA, *TRP3*, *RPS4*, and *DED1*. Quantitative PhosphorImager analysis indicates that RNA levels in mutant TBP strains were generally within ±20% of that observed in the wild-type strain. However, for unknown reasons, *TRP3* levels in the V161A strain are 50% higher than in the wild type, and tRNA levels in the N159L strain are 35% higher than in the wild type. (B) Western blot analysis using TBP antibody (α TBP) and 25 μg of protein from cell extracts of the same strains.



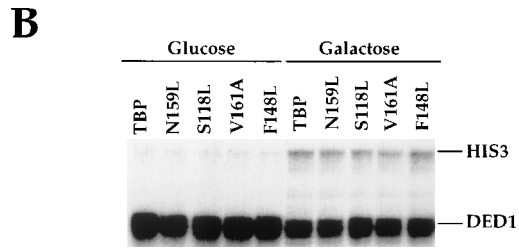


FIG. 5. Genetic complementation assay for distinguishing primary from indirect activation defects. (A) Conceptual design (see text). The absence of functional Gal4 activator (or coactivator) in the case of indirect effects is indicated by clear ovals outlined with dotted lines. (B) RNA analysis. Strains supported by wild-type and mutant TBPs were transformed with TBP^{m3}, grown in either glucose or galactose medium, and assayed for *HIS3* and *DED1* transcription.

rivatives (in an otherwise wild-type context) could support cell growth as the sole source of TBP. Of the three mutants, only L114K gave any detectable complementation of the TBP deletion; growth was extremely feeble when L114K was present on a centromeric plasmid and slow when it was introduced on a multicopy vector. When tested for activation by Gal4 and Ace1, the L114K strain was nearly as defective as the strain containing V161A (Fig. 3A and C). As a second test, all three mutants were constructed in the TBP^{m3} context and analyzed for the ability to mediate nonactivated and Gcn4-activated transcription from appropriate TGTAAA-containing promoters (Fig. 7). L114K, L189K, and K211L behaved very similarly to V161A, which was isolated in the present study.

To test whether any DNA-binding mutant could confer this phenotype, we assayed two TBP mutants (K110L and K120L) with known DNA-binding defects in vitro (55). Both of these mutants are not activation deficient by our assay; the K110L derivative behaves similarly to the parental TBP^{m3}, whereas the K120L derivative shows equivalent decreases in nonactivated and Gcn4-activated transcription (Fig. 7). These results are in accord with the previous observation that they support the response to the VP16 acidic activation domain in vitro (24). Thus, while activation deficiency in vivo correlates with mutations on the DNA-binding surface of TBP, the properties of K110L and K120L demonstrate that the converse is not true; i.e., simple disruption of TBP-DNA contacts is insufficient to cause a specific defect in activation.

DISCUSSION

TBP mutants that directly and specifically affect the response to acidic activators. We have used a novel genetic screen to isolate four TBP derivatives that are activation deficient. A key motivation for isolating the mutations in the context of TBP^{m3} was to avoid the possibility of indirect effects on transcriptional activation. Since TBP^{m3} is not required at natural TATAAA-containing promoters and is recessive to wild-type TBP in its ability to support cell growth (43), it seems highly unlikely that a mutant TBP^{m3} derivative would affect activated transcription at TGTAAA-containing promoters by altering expression of normal cellular genes. Moreover, in the genetic complementation assay (Fig. 5), the TBP^{m3} probe functioned similarly in cells supported by wild-type and activation-deficient TBP mutants. Thus, reduced transcriptional activation by Gal4 in our mutant TBP strains is a direct consequence of subnormal TBP function at the activated promoters that we assayed.

Our results strongly suggest that the four TBP derivatives are specifically impaired in the response to acidic activators. Strains supported by these TBP derivatives are defective (to various extents) for transcriptional activation by Gcn4, Gal4, and Ace1. These three activator proteins have structurally unrelated DNA-binding domains and are physiologically regu-

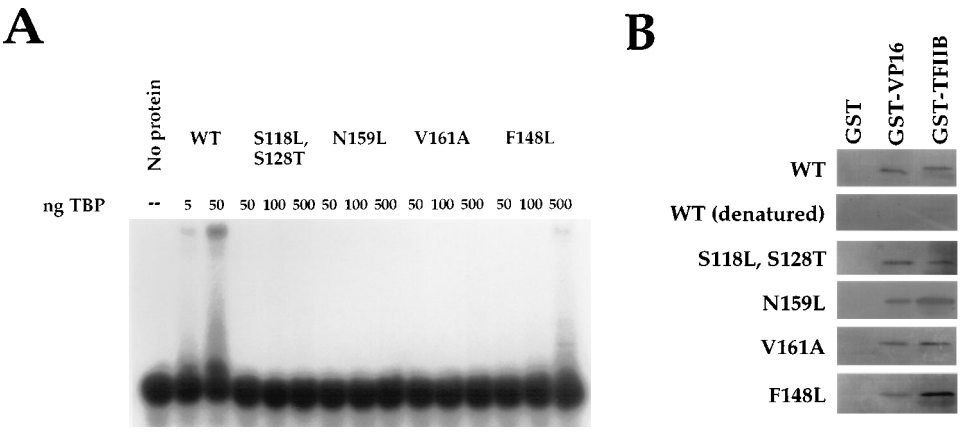


FIG. 6. In vitro analysis of TBP mutants. (A) Gel mobility shift assay for DNA binding. The indicated amount of histidine-tagged TBP protein was incubated with a TATAAA-containing probe and electrophoretically separated on a Tris-glycine 4% native polyacrylamide gel supplemented with 4 mM $MgCl_2$. WT, wild type. (B) Affinity chromatography assay for protein-protein interactions with TFIIB and VP16. Fifty nanograms of each TBP derivative was incubated with an excess of glutathione-agarose beads containing GST, GST-VP16, or GST-TFIIB. Approximately 30% of the input TBPs was retained on the columns.

lated in very distinct manners, but they all stimulate transcription through acidic activation domains (10, 16, 35). In contrast, transcription from Pol I, Pol III, and TATA-less Pol II promoters (rRNA, tRNA, and *TRP3*, respectively) is unaffected in the mutant TBP strains (Fig. 4), even though functional TBP is required in all cases in vivo (8). Furthermore, transcription from constitutive, TATA-containing Pol II promoters (*RPS4* and *DED1*) is normal in our mutant TBP strains. Although basal transcription as defined in vitro cannot be measured in vivo (see the introduction), the selective impairment of transcription mediated by three acidic activators excludes the possibility that the TBP mutations have a general effect on Pol II function.

Although these TBP mutations confer slightly reduced function on the nonactivated promoter in the TBP^{m3} context, it should be emphasized that the mutants were initially selected because of their markedly reduced ability to support activated transcription as compared with other mutants with similar competency on the nonactivated promoter. Moreover, interpretation of this weak effect on nonactivated transcription is complicated by the facts that TBP^{m3} already contains three substitutions on the DNA-binding surface and that it binds less strongly to TGTAAA than wild-type TBP binds to TATAAA (43). Thus, we conclude that the S118L, N159L, V161A, and F148L substitutions specifically affect activated transcription and that the observed effects on nonactivated transcription in the TBP^{m3} context reflect perturbations of the DNA-binding surface and TATA element interaction enhanced by the three mutations that confer altered specificity.

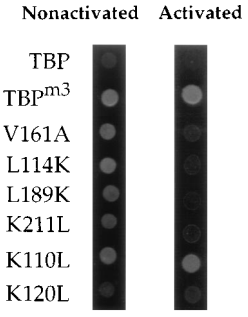


FIG. 7. Phenotypic analysis of previously characterized TBP derivatives with mutations on the DNA-binding surface of TBP. The L114K, L189K, K211L, K110L, and K120L substitutions (17, 55) were introduced into the TBP^{m3} context and examined for nonactivated and Gcn4-activated transcription (strains yML2 and yML3, respectively) as described in the legend to Fig. 2. The K120L derivative shows equivalent decreases in nonactivated and Gcn4-activated transcription (in comparison with V161A, a representative of the activation-deficient mutants isolated here, K120L has a much weaker signal on the nonactivated promoter and a slightly greater activated signal); hence, it is not considered an activation-deficient mutant.

Role of DNA binding in activation. Given that most models for transcriptional activation invoke protein-protein interactions at the promoter, we screened libraries biased against mutations on the concave, DNA-binding surface of TBP. Thus, it was quite unexpected ($P < 0.01$) that three of the four derivatives alter residues that directly interact with the TATA element. These three TBP mutants (S118L, N159L, and V161A) do not bind detectably to the TATA element in vitro, and the remaining mutant (F148L) is severely defective in DNA binding (<1% of the wild-type level). The common defect in DNA binding does not reflect gross unfolding or inactivation because all four TBP derivatives interact normally with VP16 and TFIIB in vitro.

While it is possible that these mutants are compromised in their interactions with factors that we have not tested, the localization of these mutations to the DNA-binding surface is very suggestive of the primary significance of the TBP-TATA element interaction. In support of this view, previously characterized TBP mutants that map to the DNA-binding surface and are activation defective in vitro (24) behave similarly in vivo to the mutants isolated in this paper (Fig. 7). After this report was submitted for publication, TBP mutants defective for Gal4-activated transcription in vivo were isolated by a very different genetic screen (1). These mutants also map on the DNA-binding surface of TBP and in two cases occur at the same position (although not the same substitution) as the mutants described here; however, they do not appear to be defective in activation by Gcn4 (1). Taken together, the evidence argues that mutations at the TBP-DNA surface can preferentially affect activated transcription.

Although these mutants fail to bind TATA elements in vitro, their ability to support viability indicates that they interact efficiently with these elements in vivo. This finding strongly suggests that the TBP mutants are recruited or stabilized to the TATA element in vivo, possibly by proteins (TFIIA, TFIIB,

and TAF150) that can extend the footprint of TBP on DNA in vitro (2, 49). We do not believe, however, that the activation-deficient phenotypes are due simply to a general reduction in TBP affinity for DNA. The L189K protein binds TATA sequences with wild-type affinity (55) but is activation deficient. Conversely, K110L does not bind DNA in vitro (55) but appears fully competent for activated transcription in vivo (Fig. 7) or in vitro (24). Moreover, we recently tested the four mutants isolated here in a highly sensitive assay for DNA binding, using a prebent TATA sequence (37). In this assay, V161A, the mutant most severely affected for activation, binds DNA detectably, while N159L, the least severely affected mutant, fails to bind at all (29). Taken together, the data indicate that activation deficiency cannot be explained as a simple consequence of a reduced TBP-TATA interaction.

Possible molecular mechanisms. In the simplest models, the central role of TBP in preinitiation complex formation predicts that defects in DNA binding will affect both nonactivated and activated transcription. Indeed, analysis of numerous derivatives of the TATA element suggests that the level of basal TBP-dependent transcription in vitro is very strongly (but not completely) correlated with the level of transcriptional activation in vivo (12, 52). Thus, it is very surprising to find that mutations on the DNA-binding surface of TBP can specifically affect the response to acidic activators.

Why are the TBP mutants isolated in this study specifically defective for activation? We consider four explanations that are not mutually exclusive. First, these mutants (aided by other factors such as TAFs, TFIIA, or TFIIB) might bind DNA in a conformation that differs from that of wild-type TBP such that the resulting TBP-DNA complex cannot be recognized by activators and/or coactivators. Although TBP conformation (unlike that of the TATA element) does not appear dramatically altered upon binding (23, 25), conformational differences might not need to be large to be significant and might reside in the DNA rather than in TBP. Second, activators might require that a given TBP molecule be present at the promoter for a certain length of time before they can mediate their stimulatory action. The mutant TBPs might have a slower on rate or an increased off rate from the TATA element such that an activator would not be effective. In this view, the effective occupancy time required for nonactivated transcription would be less (which might account for the lower level of transcription). Third, transcriptional activation in vivo might involve the remodelling of chromatin by factors such as the Swi/Snf complex that can enhance the accessibility of TBP for the TATA element in vitro (18). If a TBP mutant cannot exploit the remodelled chromatin generated by Swi/Snf, then activation might be greatly reduced. Fourth, the concave surface of TBP might be bifunctional such that it can interact with both DNA and activators. All of these models are consistent with the observation that only a subset of mutations on the DNA-binding surface specifically affect transcriptional activation.

Potential physiological relevance. Although the molecular mechanisms are unknown, our results indicate that interactions at the TBP-TATA interface can specifically affect the response to acidic activators. While the activation-deficient TBPs described here are artificial, our results are likely to be physiologically relevant because the affinity of TBP for natural TATA elements varies over a wide range. Indeed, there are several examples in which specific TATA sequences display differential responses to activator proteins (12, 15, 40, 44, 51). In *S. cerevisiae*, there are numerous examples of closely packed genes that are differentially regulated. In such situations, functionally distinct TATA elements might permit an activator protein that functions bidirectionally to stimulate only one of a pair of diverently transcribed genes. For example, a Gcn4-binding site is located at a comparable distance upstream of the *HIS3* and *PET56* TATA elements, but Gcn4 is able to stimulate only *HIS3* transcription (44). Thus, the subtleties involved in interactions at the TBP-TATA interface that specifically affect transcriptional activation might contribute to the extraordinary diversity of eukaryotic gene regulatory patterns.

ACKNOWLEDGMENTS

We thank Steve Buratowski for providing TBP antibody; Cammie Lesser and Christine Guthrie for *CUP1* DNAs and deletion strains; Vishwanath Iyer for Ace1-related DNAs and help with RNA analysis; Laurie Stargell for reagents and assistance with the in vitro analyses; Richard McCormick and David Fisher for prebent TATA templates; Brendan Cormack for reagents and invaluable advice in the initial stages of this work; and Susanna Ho, Sukalyan Chatterjee, Lisete Fernandes, David Dorris, and Irene Wu for critical reading of the manuscript.

This work was supported by a predoctoral fellowship to M.L. from the Howard Hughes Medical Institute and by research grants to K.S. from the National Institutes of Health (GM30186) and from the Lucille Markey Trust.

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